

The University of Chicago

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A PART OF A DISSERTATION SUBMITTED TO THE
FACULTY OF THE DIVISION OF THE BIOLOGICAL
SCIENCES IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF MEDICINE (MEDICAL CHEMISTRY)

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SULFHYDRYL ENZYMES IN CARBOHYDRATE METABOLISM

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During the last 12 years, the chemical composition of enzymes has been, to a certain extent, elucidated. It is now recognized that most of them are complex systems made up of a number of components which might be separated as individual molecular entities. Of these components, the protein moiety determines the specificity of the enzyme system; the other components can be replaced by substances of similar properties. A knowledge of the properties of these proteins, of the chemical groups essential for enzymatic activity, and of the nature of enzyme-substrate combination is therefore of fundamental importance. The protein moiety of the enzyme may be of the fibrous or of the corpuscular type; a small alteration in the spatial configuration of the protein or a small chemical change in some of the groups of the side chains might be enough to destroy enzyme activity. This is indication that there exist in the protein molecule certain bonds and certain groups, located in the side chains, without whose integrity no enzyme activity exists. Of these groups, the electronegative —SH groups have received most attention because of the ease and reliability of their detection. Of the numerous investigators who have devoted their attention to this subject, Hellerman and his coworkers, Hopkins and his coworkers, and Rapkine have made the most illuminating contribution and have inspired other workers in the search for these —SH groups among the proteins of enzyme systems. Indeed, the discovery by Hellerman *et al.* (1) of the existence in urease of sulfhydryl groups which are easily available and not necessary for enzyme activity, and others less readily available and essential for enzymatic activity, has greatly clarified many seemingly contradictory reports. The discovery by Hopkins *et al.* (2), that previous addition of malonate or succinate protects succinoxidase from inhibition by —SH inhibitors, and that of Rapkine (3), that previous addition of diphosphopyridine nucleotide protects phosphoglyceraldehyde oxidase from inhibition, have given indications for considering those —SH groups as places where union between protein and substrate or protein and prosthetic group occurs.

Up to 1938, sulfhydryl enzymes were reported only among certain hydrolytic enzymes (see the reviews by Bersin (4) and by Hellerman (5)).

The presence of sulfhydryl groups in oxidation enzymes was first demonstrated in 1938 by Hopkins and Morgan (6) in succinoxidase and by Rapkine (3) in phosphoglyceraldehyde oxidase. These findings added to those suggesting the presence of essential —SH groups in phosphoglucomutase (Lehmann (7)), hexose monophosphate oxidase (von Euler and Adler (8)), yeast alcohol oxidase (Wagner-Jauregg and Möller (9), Dixon (10)), and glycerol oxidase (Barron (11)) imply a wide distribution of the —SH enzymes (enzymes requiring the presence of certain —SH groups for enzymatic activity). In our search for the active groups of proteins responsible for enzyme activity, it was decided to start with a study of the —SH groups essential for enzymatic activity in those processes concerned with the breakdown and the synthesis of carbohydrates.

EXPERIMENTAL

Reagents for Detection of —SH Group in Enzymes—For the detection of —SH groups in enzymes use has been made of some oxidizing agents, alkylating agents, and mercaptide-forming compounds. However, since the sulfhydryl groups of proteins show different degrees of affinity for these compounds, it is preferable to submit the enzymes to the action of the three kinds of reagents before coming to the conclusion that no —SH groups are necessary for enzyme activity. The danger of using single reagents, especially those that act by oxidation, has been sufficiently emphasized by Anson (12). Three types of substances were used as a rule in our studies: iodoacetamide, *p*-chloromercuribenzoate, and trivalent organic arsenicals. As the last two reagents combine reversibly with the —SH groups, reactivation of the inactivated enzymes was attempted with either cysteine or glutathione (GSH). On some occasions alloxan, porphyrindine, iodosobenzoate, and maleate were used. The oxidation of —SH groups of proteins by alloxan was demonstrated by Labes and Freisburger (13). The use of maleate as —SH reagent was introduced by Morgan and Friedmann (14). Hellerman *et al.* (15) introduced *p*-chloromercuribenzoic acid and *o*-iodosobenzoate as —SH reagents. Iodoacetamide and porphyrindine were prepared at the laboratory; *p*-chloromercuribenzoic acid and the organic arsenicals were generously provided by Dr. L. Hellerman and by Dr. Harry Eagle. Glutathione was obtained from Schwartz Laboratories, Inc., New York.

Methods

Pyruvate was determined either manometrically with yeast carboxylase or colorimetrically by a modification of the method of Lu (16). α -Ketoglutarate was determined colorimetrically as in Lu's method with the exception that the wave-length used was 5200 Å, instead of 4200 Å as in

pyruvate. Phosphorus was determined colorimetrically by the method of Berenblum and Chain (17) at a wave-length of 7300 Å. Diphosphopyridine nucleotide was determined at a wave-length of 3450 Å. All these determinations were carried out with the Beckman photoelectric quartz spectrophotometer. Succinate was determined, after continuous extraction with ether for 4 hours, by its oxidation with pigeon breast succinoxidase. α -Ketoglutarate was determined according to Krebs (18) by oxidizing it to succinate with permanganate and subsequent extraction of the succinate. Acetoacetate was measured manometrically with aniline citrate (Edson (19)).

Succinoxidase—In 1938, Hopkins and Morgan demonstrated the presence of essential —SH groups in the activating protein (dehydrogenase) of succinoxidase. The same authors, in collaboration with Lutwak-Mann (2) found that succinate and malonate protected the —SH groups of the enzyme from —SH inhibitors. Succinoxidase was chosen for a comparative study of the inhibiting effects of a representative group of —SH reagents.

Succinoxidase was prepared as follows: Pigeon breast muscle cut in small pieces was put into a Waring blender containing 200 cc. of distilled water and an equal volume of crushed ice. After being stirred at high speed for 3 minutes, it was centrifuged for 30 minutes at 2500 R.P.M. and the supernatant fluid was discarded; this washing was repeated. The solid mass was shaken with an equal volume of 0.1 M Na_2HPO_4 for 3 hours at 30° and after centrifugation at 2500 R.P.M. for 30 minutes the supernatant fluid was used. The QO_2 of such a preparation was around 350 at pH 7.4 and 38°. It contained no lactic or malic dehydrogenases. In Table I are given the relative degrees of inhibition of succinoxidase by arsenicals, *p*-chloromercuribenzoate, arsenite, iodoacetamide, iodoacetate, and three oxidizing agents used for the determination of —SH groups in proteins; namely, ferricyanide, porphyrindine, and *o*-iodosobenzoate. The arsenicals and *p*-chloromercuribenzoate had about the same combining power for the —SH groups of the protein, as is clearly demonstrated in Fig. 1, where the degree of inhibition was plotted against the concentration of the inhibitors. 50 per cent inactivation was obtained with 3.2×10^{-5} M *p*-chloromercuribenzoate and with 3.15×10^{-5} M 3-amino-4-hydroxyphenyldichloroarsine hydrochloride. The affinity of these compounds for the —SH groups of the enzyme was much greater than the affinity of the enzyme for succinate and for malonate (with succinate concentration at 5×10^{-2} M). With increasing concentrations of succinate, 50 per cent activity was reached with 200×10^{-5} M. In the presence of 0.05 M succinate, 125×10^{-5} M malonate produced 50 per cent inhibition.

Of the oxidizing agents used (at equimolecular concentrations) *o*-iodosobenzoate was the most powerful inhibitor, porphyrindine came next, while

ferricyanide had a low inhibiting action at this concentration. This explains why ferricyanide could be used by Quastel and Wheatley (20) as the oxidizing agent of succinate when its oxidation by succinoxidase was performed in absence of oxygen.

Of the alkylating agents, iodoacetate has been extensively used, although Michaelis and Schubert (21) called attention to the combination of acid halides with $-\text{NH}_2$ groups. This possibility is usually dismissed as occurring in enzyme inhibitions, because it is assumed that in neutral solutions iodoacetate combines only with $-\text{SH}$ groups. If this assumption is correct, once all the $-\text{SH}$ groups necessary for activity are covered, say by mercap-

TABLE I

Effect of —SH Reagents on Succinoxidase

Enzyme, from pigeon breast muscle; pH 7.4 (0.033 M PO_4); succinate, 0.02 M; 38°; duration of experiments, 20 minutes; QO_2 of a typical preparation, 351 c.mm. For convenience the arsenicals are called by their numbers in the text.

Inhibitor	Concentration	Inhibition
	<i>M</i>	<i>per cent</i>
<i>p</i> -Carboxyphenylarsine oxide (I)	4.2×10^{-5}	79.3
<i>p</i> -Carbamylphenylarsine " (II)	4.2×10^{-5}	80
<i>p</i> -Aminophenyldichloroarsine hydrochloride (III)	4.2×10^{-5}	80.5
3-Amino-4-hydroxyphenyldichloroarsine hydrochloride (IV)	4.2×10^{-5}	51.3
Phenylarsine oxide (V)	4.2×10^{-5}	78.1
Sodium arsenite	5×10^{-3}	15
Iodoacetamide	5×10^{-3}	64
<i>p</i> -Chloromercuribenzoate	5×10^{-5}	73.5
Iodoacetate	1×10^{-3}	42
Ferricyanide	5×10^{-4}	28
<i>o</i> -Iodosobenzoate	5×10^{-4}	Complete
Porphyrindine	5×10^{-4}	90

tide formation, iodoacetate ought to have no action on the enzyme. Addition of a thiol in concentration sufficient to combine with iodoacetate and to dissociate the mercaptide ought to produce reactivation of the enzyme to the same extent as reactivation after inhibition with the mercaptide-forming compound above. (GSH does not reverse the combination of the protein with iodoacetate.)

To test this assumption, samples of succinoxidase were inhibited by *p*-chloromercuribenzoate to complete inhibition, by iodoacetate to 86 per cent inhibition, and by iodoacetamide to 80 per cent inhibition. To other vessels containing enzyme plus *p*-chloromercuribenzoate there were added 10 minutes later iodoacetate and iodoacetamide. At the end of 35 minutes

the reactivation of the enzyme was performed with glutathione. The enzyme inhibited by *p*-chloromercuribenzoate alone was 84 per cent reactivated by glutathione; in the presence of *p*-chloromercuribenzoate plus iodoacetate, there was only 13 per cent reactivation; in the presence of iodoacetamide plus *p*-chloromercuribenzoate the reactivation reached 68 per cent. These experiments show that iodoacetate and iodoacetamide may produce enzyme inhibition by a mechanism other than combination with —SH groups, the first to a large extent, the second to a smaller degree (Table II).

Hopkins *et al.* (2) on reporting the inhibition produced by oxidized glutathione emphasized that the inhibition was a slow process reaching its maximum intensity gradually. Such was not the case in the inhibition by

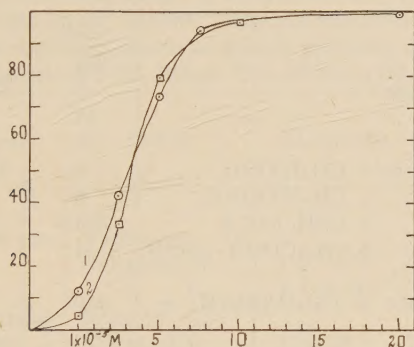


FIG. 1. Inhibition of succinoxidase by *p*-chloromercuribenzoate and organic arsenicals. Abscissa, concentration of inhibitor, $\times 10^{-5}$ M; ordinate, per cent inhibition. Curve 1, *p*-chloromercuribenzoate; Curve 2, 3-amino-4-hydroxyphenyldichloroarsine hydrochloride. 1 cc. of enzyme; 1 cc. of phosphate buffer, 0.066 M, pH 7.2; 0.25 cc. of 0.25 M succinate; total volume, 2.5 cc.

arsenicals, in which maximum inhibition was attained 6 minutes after addition of the arsenical. While the oxidation of —SH groups was a slow process, the formation of thioarsenite was rather quick.

The very interesting observation of Hopkins *et al.* (2) on the protection by malonate against inhibition by oxidized glutathione has also been noted when arsenicals were used as inhibitors. 7.5×10^{-5} M Arsenical IV produced in these experiments 73 per cent inhibition when added before the succinate; 2.5×10^{-3} M malonate produced an inhibition of 43 per cent. When malonate and arsenical were added together, the O_2 uptake was almost the same as when only malonate was present (Table III). Potter and DuBois (22) have also repeated these experiments of Hopkins *et al.*, using quinone as inhibitor, and have confirmed this protective action of malonate.

Enzymes for Pyruvate Metabolism—It is now recognized that pyruvate, the end-product of the first phase of carbohydrate fermentation (23), is extremely reactive and undergoes decarboxylation, oxidation, dismutation, condensation, and CO₂ fixation. Whether all these reactions are catalyzed

TABLE II

Effect of Iodoacetate and Iodoacetamide on Succinoxidase Treated with p-Chloromercuribenzoate. Reactivation by Glutathione

p-Chloromercuribenzoate, 5×10^{-4} M; CH₂ICOOH, 3×10^{-2} M; CH₂ICOOH₂, 5×10^{-3} M; succinate 5×10^{-2} M; buffer, phosphate, 0.03 M, pH 7.2. Succinoxidase treated with those reagents either alone or in combination for 35 minutes at 23°. Enzyme activity tested at 38°.

System	O ₂ uptake in 30 min.	Inhibition	Reactivation
	<i>c.mm.</i>	<i>per cent</i>	<i>per cent</i>
Control	395		
p-Chloromercuribenzoate	0	Complete	
CH ₂ ICOOH	54	86	
CH ₂ ICOOH ₂	81	80	
p-Chloromercuribenzoate + CH ₂ ICOOH	0	Complete	
“ + CH ₂ ICOOH ₂	0	“	
“ + GSH, 0.01 M	333.5	15.5	84.5
“ + CH ₂ ICOOH + GSH,	51	87	13
5.3 × 10 ⁻² M			
p-Chloromercuribenzoate + CH ₂ ICOOH ₂ +	250	32	68.4
GSH, 4.7 × 10 ⁻² M			

TABLE III

Protective Action of Malonate against Arsenical Inhibition of Succinoxidase (Hopkins Phenomenon)

System	O ₂ uptake in 20 min.	Inhibition
	<i>c.mm.</i>	<i>per cent</i>
Succinate, 0.05 M	318.7	
“ and 7.5×10^{-5} M Arsenical IV	85.5	73.2
“ “ 2.5×10^{-3} “ malonate	179.6	43.7
“ “ malonate (2.5×10^{-3} M) and Arsenical IV	169.7	46.7
(7.5×10^{-5} M)		

by the same enzyme or by different enzymes has not yet been established, although there is more evidence in favor of the second assumption. The search for —SH groups among these enzyme systems was made by studying the different reactions in which pyruvate is one of the reactants under the optimum conditions for each reaction. The —SH nature of pyruvate oxi-

dase was first suggested by Peters (24), and some evidence in favor of this suggestion is found in Barron's paper on α -ketonoxidase from gonococci (25). Pyruvate oxidation was found quite sensitive to atmospheric oxygen, and was inhibited by quinone at low concentrations (90 per cent inhibition with 3×10^{-5} M quinone). Snell and Weissberger (26) 3 years later presented evidence for the formation of addition compounds between quinone and thiols.

The effect of sulfhydryl reagents on *pyruvate oxidation* was studied in the enzyme present in gonococci, in ground, washed avian liver, and in rat liver

TABLE IV
Effect of —SH Reagents on Pyruvate Oxidation

Experiment I, gonococci suspension in Ringer-PO₄ solution; pH 7.4; pyruvate concentration, 0.02 M; gas phase, air; 38°. Experiment II, Ringer-PO₄ solution; pH 7.4; Mg⁺⁺, Mn⁺⁺, and diphosphothiamine added; pyruvate concentration, 0.02 M; gas phase, air; 38°. Experiment III, Ringer-PO₄ solution; pH 7.4; gas phase, O₂; 38°.

Experiment No.	Source of enzyme	Inhibitor	Concentration	O ₂ uptake or pyruvate utilization per mg.	Inhibition
			M	c.mm.	per cent
I	Gonococci (O ₂ uptake)	None		298	
		Arsenical IV	1×10^{-4}	18	94
		" I	1.1×10^{-4}	0	Complete
II	Washed, ground chicken liver (O ₂ uptake blank subtracted)	None		220.5	
		Arsenical I	1×10^{-4}	0	Complete
		<i>p</i> -Chloromercuribenzoate	5×10^{-4}	17.2	92
		Iodoacetamide	5×10^{-3}	0	Complete
III	Rat liver slices (pyruvate utilization)	None		72.6	
		Arsenical III	5×10^{-4}	18.1	75

slices. In the first two, O₂ uptake was measured (gonococci take up no oxygen in the absence of pyruvate and the residual oxygen uptake of washed liver is small); in rat liver, the pyruvate utilization was determined. The oxidation of pyruvate by gonococci was inhibited completely by Arsenicals IV and I; the oxidation by ground chicken liver (prepared as described by Barron *et al.* (27)) was inhibited by Arsenical I, *p*-chloromercuribenzoate, and iodoacetamide. The utilization of pyruvate by rat liver slices was inhibited by Arsenical III (75 per cent) (Table IV).

The *dismutation of pyruvate* ($2\text{CH}_3\text{COCOOH} \rightarrow \text{CH}_3\text{CHOHCOOH} + \text{CH}_3\text{COOH} + \text{CO}_2$) was studied in minced pigeon liver suspended in Ringer-bicarbonate solution, with N₂-CO₂ as the gas phase. The CO₂ formation

and pyruvate utilization were determined manometrically. The inhibiting effect of the —SH reagents was not as marked as in pyruvate oxidation. In fact, concentrations of arsenicals, *p*-chloromercuribenzoate, and iodoacetamide which produced complete inhibition of pyruvate oxidation gave only partial inhibition of pyruvate dismutation. However, 1×10^{-3} M *p*-chloromercuribenzoate and 5×10^{-3} M iodoacetamide inhibited this reaction completely. The formation of acetoacetate from pyruvate was studied in ground pigeon liver in the presence of malonate (1×10^{-2} M). Arsenical III (2×10^{-3} M), *p*-chloromercuribenzoate (1×10^{-3} M), and iodoacetamide (5×10^{-3} M) inhibited completely this pyruvate condensation reaction. The formation of acetylmethylcarbinol was studied with the enzyme prepared from heart according to Green *et al.* (28), by determining the CO₂ formation manometrically in the presence of pyruvate and acetaldehyde, in N₂-CO₂ as gas phase. This reaction was inhibited 46 per cent by 1×10^{-3} M Arsenical IV, 85 per cent by 1×10^{-3} M *p*-chloromercuribenzoate, and 64 per cent by 5×10^{-3} M iodoacetamide. The formation of α -ketoglutarate (CO₂ fixation?) was studied with pigeon liver extract, prepared according to Evans and Slotin (29). The enzyme, extracted with NaCl-bicarbonate saturated with O₂-CO₂ (pH 7.4), was incubated with 5×10^{-2} M pyruvate and 6.6×10^{-3} M malonate (O₂-CO₂ as gas phase). Arsenical I (5×10^{-4} M) inhibited the reaction 73 per cent; iodoacetamide (5×10^{-3} M), 74 per cent; maleate (2.5×10^{-3} M), 26 per cent.

J. Meyer, of this laboratory, found (unpublished experiments) that *yeast carboxylase* was inhibited by the —SH reagents iodoacetate, iodoacetamide, iodine, CuCl₂, *p*-chloromercuribenzoate. He observed, furthermore, that the extent of inhibition was greater when the —SH reagent was added to carboxylase in the absence of cocarboxylase (alkaline washed yeast), an indication that the prosthetic group was attached to the protein at the side chains of the —SH groups, and in agreement with Rapkine's observation on phosphoglyceraldehyde oxidase and protection with pyridine nucleotide. Further evidence that carboxylase is an —SH enzyme was obtained by inhibition with Arsenical III (Table V). Greenberg and Rinehart (30) offered indirect evidence of the necessity of —SH groups for enzyme activity of carboxylase in experiments in which the activity of alkaline washed yeast was increased on addition of thiol compounds (glutathione and cysteine).

α -Ketoglutarate Oxidase—Krebs and Johnson (31) found that in the presence of arsenite minced pigeon breast muscle converts quantitatively citric acid into α -ketoglutaric acid. The experiments presented in Table VI show that this inhibition of α -ketoglutarate oxidation by arsenite (in the absence of arsenite, α -ketoglutarate is oxidized by minced pigeon breast muscle) is due to a combination with essential —SH groups of the activating protein of the enzyme. The oxidation of α -ketoglutarate by kidney slices in the

TABLE V

Effect of —SH Reagents on Enzyme Systems for Pyruvate Metabolism

Experiment I, ground, washed pigeon liver; buffer, NaCl-NaHCO₃, pH 7.4; pyruvate, 0.02 M (Mn⁺⁺, Mg⁺⁺, and diphosphothiamine added); gas phase, N₂-CO₂; 38°. Experiment II, ground liver suspended in NaCl-phosphate, pH 7.4; malonate, 0.02 M; pyruvate, 0.02 M; air as gas phase; 38°. Experiment III, enzyme from pig heart suspended in phosphate-bicarbonate, pH 6.0; gas phase, N₂-CO₂; 38°. Experiment IV, enzyme extracted from pigeon liver, suspended in Ca-free bicarbonate-Ringer's solution, pH 7.4; pyruvate, 0.04 M; malonate, 0.066 M; gas phase, O₂-CO₂; 38°. Experiment V, washed brewers' yeast, 30 mg. per vessel; acetate buffer, pH 4.81; 25°.

Ex- peri- ment No.	Enzyme system	Inhibitor	Concentration	Reaction expressed as c.mm. O ₂ uptake, pyruvate utilization, or formation of acetoacetate and α -ketoglutarate	Inhibition
			M		per cent
I	Pyruvate dismuta- tion (anaerobic pyruvate utiliza- tion)	None		14.1	
		Arsenical III	5×10^{-4}	9.1	35.5
		p-Chloromer- curibenzoate	5×10^{-4}	10.0	29.0
		Iodoacetamide	5×10^{-4}	12.1	14.2
		p-Chloromer- curibenzoate	1×10^{-3}	0	Complete
II	Pyruvate condensa- tion (acetoacetate formation)	Iodoacetamide	5×10^{-3}	0	"
		None		147.4	
		Arsenical III	2×10^{-3}	0	Complete
		p-Chloromer- curibenzoate	1×10^{-3}	0	"
III	Pyruvate condensa- tion (acetylmethyl- carbinol forma- tion) determined by CO ₂ release	Iodoacetamide	5×10^{-3}	0	"
		None		230.6	
		Arsenical IV	1×10^{-3}	124.2	46
		p-Chloromer- curibenzoate	1×10^{-3}	33.7	85.4
IV	Pyruvate CO ₂ fixation (α -ketoglutarate formation)	Iodoacetamide	5×10^{-3}	82.0	64.5
		None		1785	
		Arsenical	5×10^{-4}	478	73.2
		Maleate	2.5×10^{-3}	1320	26.0
V	Pyruvate decarboxy- lation	Iodoacetamide	5×10^{-3}	460	74.0
		None		115	
		p-Chloromer- curibenzoate	1×10^{-3}	0	Complete
		Iodoacetamide	5×10^{-3}	31	73
	Yeast carboxylase* (CO ₂ formation)*	Iodoacetate	5×10^{-3}	46	60
		None		208	
		Arsenical III	1.2×10^{-3}	21.8	89

* Values obtained by Mr. J. Meyer in alkaline washed yeast.

presence of malonate was inhibited by the three most specific reagents for —SH group detection, organic arsenical, *p*-chloromercuribenzoate, and iodoacetamide.

Malate Oxidase—Green (32) in his paper on malic dehydrogenase reports that 38 per cent of the activity of malate oxidase was lost on treatment with 0.03 M iodoacetate, and that 0.03 M arsenite ("arsenious acid") and 0.03 M maleate had no effect. Evidence that the activating protein of this enzyme belongs to the group of —SH enzymes is given in Table VII. The activating protein (dehydrogenase) of malate oxidase, prepared according to Green, was treated with organic arsenical, *p*-chloromercuribenzoate, and iodoacetamide, enzyme activity being measured by the O₂ uptake in the presence of diphosphopyridine nucleotide and semicarbazide. (Sufficient amounts of alloxazine dinucleotide and cytochrome-cytochrome oxidase exist in the

TABLE VI

Inhibition of α-Ketoglutarate Oxidase by —SH Reagents

Rat kidney slices in Ringer-phosphate solution containing 0.02 M malonate. The inhibitors were left in contact with the tissue for 20 minutes previous to addition of α-ketoglutarate. α-Ketoglutarate, 0.01 M. The figures are given in c.mm. per mg. of dry tissue in 2 hours.

Inhibitor	O ₂ uptake		Inhibition	α-Ketoglutarate utilization		Inhibition
	Control	Inhibitor		Control	Inhibitor	
	c.mm.	c.mm.	per cent	c.mm.	c.mm.	per cent
Arsenical V, 1×10^{-4} M	13.6	3.8	72	78.2	1.3	98
<i>p</i> -Chloromercuribenzoate, 2.5×10^{-4} M	14.7	4.4	70			
Iodoacetamide, 2.0×10^{-3} M	14.7	0.6	96			

enzyme suspension to allow a steady O₂ uptake.) *p*-Chloromercuribenzoate at a concentration of 2×10^{-4} M inhibited completely the O₂ uptake; the same concentration of Arsenical V produced an inhibition of 51 per cent, while 3×10^{-3} M iodoacetamide had no effect at all. The —SH groups of this enzyme, so readily destroyed by Hellerman's reagent, resisted somewhat the action of arsenicals and were not acted upon by iodoacetamide (Table VII).

Alcohol Oxidase—In 1935 Wagner-Jauregg and Möller (9) found that CuSO₄ inhibited alcohol oxidase, the inhibition being reversed by glutathione. This observation added to that of Dixon (10) of inhibition by iodoacetate indicates that the enzyme is an —SH protein. Further evidence in support of this contention is given in Fig. 2. The activating protein of alcohol oxidase, prepared according to Negelein and Wulff (33), was inhibited completely by 5×10^{-4} M *p*-carboxyphenylarsine oxide and partially

by 2.5×10^{-4} M. In contrast to this powerful inhibition, 5×10^{-4} M arsenite had very little effect on the rate of reduction of diphosphopyridine nucleotide.

Lutwak-Mann (34) made the interesting discovery that while yeast alcohol dehydrogenase was readily inhibited by iodoacetate, liver alcohol dehydrogenase was not affected by this reagent. Lutwak-Mann's findings were confirmed by studying the rate of reduction of diphosphopyridine nucleotide by alcohol, activated by liver alcohol dehydrogenase (in the presence of 1×10^{-3} M semicarbazide to bind the acetaldehyde formed during the oxidation of alcohol). The rate was unaffected by the addition of organic arsenicals, *p*-chloromercuribenzoate, and iodoacetamide. The inhibition of yeast alcohol oxidase by —SH reagents and the ineffectiveness on liver alcohol oxidase show that, while the protein of the alcohol oxidase

TABLE VII

Inhibition of Malate Oxidation by —SH-Combining Substances

Enzyme from pigeon heart, 3.0 cc.; malate, 1 M, 0.3 cc.; semicarbazide, 0.1 M, 0.5 cc.; diphosphopyridine nucleotide (0.2 per cent), 0.5 cc.; 0.1 M phosphate, pH 7.4, enough to make 4.95 cc.; gas phase, air; 38°. The blank gave no O₂ uptake.

Inhibitor	Concentration	O ₂ uptake		Inhibition
		Control	Inhibitor	
	M	c.mm.	c.mm.	per cent
Arsenical I	5×10^{-4}	105.2	32.1	69.5
“ V	2×10^{-4}	85.2	41.8	51.0
<i>p</i> -Chloromercuribenzoate	2×10^{-4}	85.2	0	Complete
Iodoacetamide	3×10^{-3}	109.0	109.4	None

from yeast required —SH groups for enzyme activity, the protein of the same enzyme from liver needs no —SH groups.

Effect of —SH Reagents on Other Protein Components of Oxidation Enzymes—It is known that most of the oxidation enzymes which take part in carbohydrate metabolism are complex systems made up of an activating protein (dehydrogenase) with which a prosthetic group might be combined, and oxidation-reduction systems (flavins, iron porphyrins) united to proteins. Thiol groups essential for enzyme activity, reported previously and those reported in this paper, have been found exclusively in activating proteins. The activity of cytochrome oxidase, as determined by the rate of oxidation of reduced cytochrome *c* was completely unaffected by *p*-chloromercuribenzoate, arsenicals, and iodoacetamide. The activity of heart flavoprotein (flavin dinucleotide) as tested with the activating protein of lactate oxidase, diphosphopyridine nucleotide, and methylene blue was also found un-

affected on addition of the same reagents. The activity of catalase was also unaffected by the addition of —SH reagents.

Furthermore, enzymes of less complex composition (metalloproteins) were also found to require no —SH groups for enzyme activity. Among these enzymes, uricase, polyphenol oxidase, carbonic anhydrase, and phosphatases were not affected by the addition of —SH reagents.

Lactic and isocitric dehydrogenases were also found not to be affected in their activity on addition of —SH reagents. The first enzyme was prepared from heart, the rate of diphosphopyridine nucleotide reduction being meas-

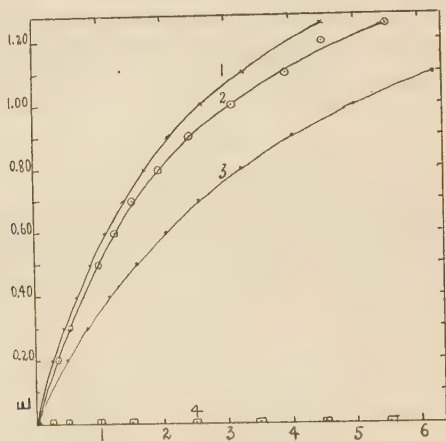


FIG. 2. The inhibition of yeast alcohol oxidase (activating protein) by organic arsenicals. 0.1 cc. of activating protein (20 γ of enzyme of 28 per cent purity); 2 mg. of diphosphopyridine nucleotide of 40 per cent purity; 0.003 M semicarbazide; pyrophosphate buffer, 0.03 M, pH 7.9; total volume, 4 cc. E , extinction coefficient at 3450 A (ordinate); abscissa, time in minutes. Curve 1, control; Curve 2, 5×10^{-4} M sodium arsenite; Curve 3, 2.5×10^{-4} M *p*-carboxyphenylarsine oxide; Curve 4, 5×10^{-4} M *p*-carboxyphenylarsine oxide.

ured. The second enzyme was prepared from liver; here the rate of methylene blue reduction was the measure of enzyme activity with citrate as substrate.

Reactivation of —SH Enzymes—When the —SH groups of the —SH enzymes are rendered inactive either by oxidation or mercaptide formation, they may under certain conditions be regenerated with complete reactivation of enzyme activity by the addition of reducing agents (ascorbic acid, H_2S , cyanide) or mercaptide-forming substances (thiols). In this last case the degree of reactivation is determined, among other factors, by the affinity of the enzyme and of the thiol to the mercaptide-forming inhibitor (dissociation constants of the respective mercaptides). Inhibitor and reac-

tivator compete for the —SH groups. If the competitor has greater affinity for the inhibitor than the enzyme, or is used in large concentrations, reactivation of the enzyme will result.

Reactivation of the —SH enzymes described in this paper was attempted with either glutathione or cysteine in concentrations about 10 times that of the —SH inhibitor. As a rule the thiol compounds were added 10 minutes after addition of the inhibitor, time sufficient to produce maximum inhibition of the enzyme system. In every case blanks were run with enzyme plus thiol. Succinoxidase, inhibited by organic arsenicals or quinone, was completely reactivated on addition of glutathione (50 times the concentration of the inhibitor) (Table VIII). This reactivation produced by glutathione was instantaneous and in no way connected with its oxidation by metal catalysts in the enzyme, since the enzyme suspension showed no appreciable O_2 uptake on addition of glutathione without succinate during the course of the experiments (20 minutes).

Enzymes for Pyruvate Metabolism—Before the —SH nature of pyruvate oxidase was demonstrated, it was found that pyruvate oxidation and pyruvate condensation by washed pigeon liver were considerably increased on addition of glutathione (35). Undoubtedly, during the process of grinding and washing, the —SH groups of the enzyme were partially oxidized either by atmospheric oxygen or heavy metal impurities. The oxidation of pyruvate by gonococci, 94 per cent inhibited by Arsenical IV (1×10^{-4} M), was reactivated 15 per cent on addition of glutathione (Table VIII). When the arsenical concentration was diminished so as to produce only partial inhibition (40 per cent), the reactivation by glutathione increased to 77 per cent. The oxidation of pyruvate by ground washed chicken liver, inhibited by *p*-chloromercuribenzoate, was partially reactivated by glutathione (39 per cent reactivation). The oxidation of pyruvate by rat liver slices, inhibited by Arsenical III (75 per cent inhibition), was reactivated by glutathione (97 per cent), with a concentration 10 times that of the arsenical. The inhibition of yeast carboxylase by Arsenical III was abolished on addition of cysteine and H_2S (8 times the inhibitor concentration); the inhibition produced by *p*-chloromercuribenzoate was abolished on addition of glutathione. Similar reactivation of the —SH enzymes for pyruvate condensation (acetoacetate synthesis, acetylmethylcarbinol formation) was observed on addition of glutathione, about 10 times the concentration of the inhibitor. Pyruvate dismutation was only partially reactivated even when the inhibitor concentration was lowered so as to give only 35 per cent inhibition. There were technical difficulties when reactivation of α -ketoglutarate formation was attempted, because the determination of α -ketoglutarate (oxidized to succinate) was performed with the aid of succinoxidase. When the succinate was extracted with ether, there appeared to be also extraction of the sulphydryl inhibitor.

TABLE VIII
Reactivation of —SH Enzymes Inhibited by Mercaptide-Forming Substances

The conditions of the experiments were similar to those described in the preceding tables.

Enzyme	Inhibitor	Inhibition <i>per cent</i>	Reactivator	Reactivation <i>per cent</i>
Succinoxidase	Arsenical I, 5×10^{-6} M “ “ 5×10^{-5} “	80 80	Glutathione, 1×10^{-3} M Cysteine, 1×10^{-2} M	Complete 25
Pyruvate oxidase (gonococci)	Benzoquinone, 5×10^{-6} M Arsenical IV, 1×10^{-4} M “ “ 1.5×10^{-5} “	80 94 40	Glutathione, 2×10^{-3} M “ 1×10^{-3} “ “ 1×10^{-3} “	Complete 15 77.5
“ “ (ground, washed chicken liver)	<i>p</i> -Chloromercuribenzoate, 5×10^{-4} M	92	“ 2.5×10^{-3} M	39
Pyruvate oxidase (liver slices)	Arsenical III, 5×10^{-4} M	75	“ 5×10^{-3} M	97
Yeast carboxylase	“ “ 1.2×10^{-3} M “ “ 1.2×10^{-3} “ <i>p</i> -Chloromercuribenzoate, 1×10^{-3} M “ “ 1×10^{-3} “	89.5 89.5 95	Cysteine, 1×10^{-2} M H_2S , 1×10^{-2} M Glutathione, 1×10^{-2} M	72 97 92
Pyruvate condensation (ace- tylmethylcarbinol forma- tion)	Arsenical IV, 1×10^{-3} M	85 46	“ 1×10^{-2} “ “ 1×10^{-2} “	Complete “
Pyruvate condensation (ace- toacetate synthesis)	“ “ 1×10^{-3} “ <i>p</i> -Chloromercuribenzoate, 1×10^{-3} M	Complete “	“ 5×10^{-3} “ “ 1×10^{-2} “	“ 36
Pyruvate dismutation	Arsenical III, 5×10^{-4} M	35	“ 5×10^{-3} “	54
Malate oxidase	“ “ 1×10^{-4} M	69	“ 5×10^{-3} “	71
Adenosinetriphosphatase (myosin)	<i>p</i> -Chloromercuribenzoate, 1×10^{-3} M	Complete	“ 1×10^{-2} “	Complete

Malate oxidase inhibited by Arsenical I was reactivated by glutathione at a concentration 10 times that of the inhibitor. It has been shown that adenosinetriphosphatase (myosin) is an —SH enzyme (36). This enzyme inhibited by *p*-chloromercuribenzoate was completely reactivated on addition of glutathione (Table VIII).

The —SH groups are essential not only for the enzymatic activity, but also for the stability of the protein. When the sulfhydryl groups have been destroyed by oxidation or mercaptide formation, complete reactivation of the enzyme is possible only within certain time limits, beyond which the protein loses enzyme activity irreversibly. This must be the reason for the partial reactivations. Succinoxidase, for example, completely inhibited by *p*-chloromercuribenzoate, was completely reactivated by glutathione when the latter was added 10 to 15 minutes after the mercurial; 85 per cent reactivated when added 30 minutes after the inhibitor; 70 per cent reactivated when added 60 minutes later.

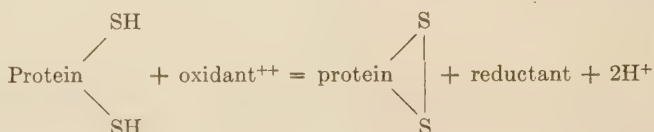
DISCUSSION

It is known that the —SH groups of amino acids possess a certain resistance to the action of mild oxidizing agents, possibly because of the formation upon oxidation of the —S—S— bond. In the protein molecule this resistance may be increased by a variety of factors: the position of the —SH groups with respect to each other, a physical factor which affects not only the rate of oxidation but also the possibility of oxidation; the electronegativity values of the groups neighboring the —SH groups; steric hindrances of various sorts. In some instances the —SH groups are so situated that these factors contribute to the loosening of the —SH bond, ready oxidation being the consequence. In other cases these factors add up to the tightening of the bond and make oxidation impossible unless by rupture of some H bonds in the protein molecule (denaturation) the hindering factors have been abolished. For convenience we may then divide the protein —SH groups into two types, *freely reactive —SH groups*, which are oxidized and give the nitroprusside test when the protein is in the native state, and *sluggish —SH groups*, which are not oxidized and do not give the nitroprusside test except after denaturation of the protein.

In general, the oxidizing agents usually employed for the titration of these —SH groups (ferricyanide, porphyrindine) react only with those present in the native protein; sometimes even some of these —SH groups fail to react. The alkylating agents react with the —SH groups as second order reactions (Hellström (37)), and large concentrations are required for complete reaction; they react also with —NH_2 groups, especially iodoacetate. The mercaptide-forming compounds seem to possess a greater affinity for —SH groups, as they attack those that have been left untouched by the oxidizing

agents. The affinity of these sulfhydryl groups for the —SH reagents varies from protein to protein, depending among other factors on their position in the molecule and their degree of dissociation. In some instances only certain —SH groups are essential for enzyme activity, as Hellerman found in urease. It is essential to have these facts in mind when detecting —SH enzymes; otherwise erroneous conclusions may be reached. Reagents must be selected that do not produce protein denaturation and that react with all types of —SH groups; in the case of mercaptide formation reactivations must be produced. It is only when various selected reagents have produced enzyme inhibition that satisfactory conclusions can be reached.

The oxidizing agents so widely used for the titration of —SH groups in proteins have proved to be less effective in detecting —SH enzymes. They oxidize the —SH groups to the disulfide,

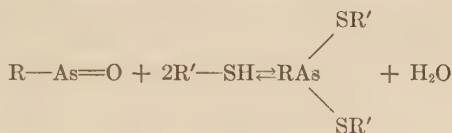


thus requiring the presence of —SH groups close enough for bond formation. There may be native protein molecules in which, due to the particular stereochemical structure, the individual —SH groups are not sufficiently close together to form an S—S bond. In these cases oxidizing agents might be ineffective in detecting —SH groups. For example, ferricyanide, used by Anson (38) and by Mirsky (39) for the titration of —SH groups in proteins, has no effect on the enzymatic activity of urease (Hellerman (5)) and of choline oxidase, enzymes requiring the presence of —SH groups for activity, and has weak inhibitory action on succinoxidase. Porphyrindine, the reagent used by Greenstein (40), has no effect on the activity of papain, an —SH enzyme (41).

Iodoacetate has been considered a valuable and almost specific reagent for the detection of —SH enzymes. When acting on proteins it is assumed that it reacts by substitution of the H of —SH by the carboxymethyl group, $\text{protein-SH} + \text{CH}_2\text{ICOOH} = \text{protein-SCH}_2\text{COOH} + \text{HI}$ (Rapkine (42), Dickens (43)). Under certain conditions this alkylation of —SH groups is more rapid than that of amino groups but reaction with —SII groups does not preclude reaction with —NH₂ groups, as has been shown in experiments in which *p*-chloromercuribenzoate and iodoacetate were added together to succinoxidase. Moreover, Smythe (44) has shown the great variation in the rate of interaction of iodoacetate with even simple alkyl mercaptans. Even iodoacetamide (a better —SH reagent in this group) is a poor reagent for the detection of —SH groups because of the irregularity of its action.

(It does not react with the —SH groups of egg albumin at pH 7.3 (Rosner (45)), while it abolishes 40 per cent of them at pH 9 (Anson (46).) Similar variations are found when iodoacetamide is used for detecting —SH enzymes. At a concentration 3 times higher than that required for abolition of the active —SH groups of urease, iodoacetamide had no effect on the enzyme activity (Hellerman *et al.* (1)). It had no effect on the enzyme activity of malate oxidase or of adenosinetriphosphatase, both enzymes requiring essential —SH groups.

The third group of reagents consists of those which form addition compounds. The trivalent organic arsenicals used in the experiments reported here belong to this group. They combine reversibly with —SH groups (Voegtlin, Dyer, and Leonard (47), Cohen, King, and Strangeways (48)),



giving compounds which readily dissociate on increase of the pH value of the solution, or when the pH is kept constant, on addition of another thiol compound possessing greater affinity for the arsenical. They do not react with the —SH groups of native egg albumin but combine with them on denaturation (Rosenthal (49)). They were used first as enzyme inhibitors by Bersin (50). The organic arsenicals are powerful tools in the quest for —SH groups in enzymes not only because they form reversible thioarsenites but also because their power to link with —SH groups approaches that of Hellerman's *p*-chloromercuribenzoate.

From the effect of the inhibitors on the enzyme systems reported here, a rough classification could be made of the —SH reagents: (1) mild oxidizing agents (ferricyanide, porphyrindine, 2,6-dichlorophenol indophenol) which act only on those —SH groups close enough to allow S—S formation; (2) certain alkylating reagents (iodoacetamide), which, though combining with more —SH groups than the former, leave some unattacked; (3) reagents like arsenicals and *p*-chloromercuribenzoate which combine with all the available —SH groups in the native protein. Of all the enzyme systems studied, pyruvate oxidase and the enzyme for pyruvate condensation (acetoacetate formation) were the most sensitive to the action of enzyme inhibitors; in fact, all three classes of reagents produced enzyme inhibition. Succinoxidase and most of the other —SH enzymes are not significantly inhibited by low concentrations of ferricyanide; they are inhibited by the second and third class of —SH reagents. Finally urease is not inhibited by mild oxidizing agents or arsenicals; the active sulphydryl groups are abolished only by the powerful —SH reagent, *p*-chloromercuribenzoate. These

examples show that a step by step abolition of the —SH groups of proteins can be accomplished by the use of reagents possessing different affinities for these groups.

The experiments presented here, as well as those found in the literature, demonstrate that the presence of —SH groups is essential for the activity of a large number of enzymes concerned with the metabolism of carbohydrate. In the first phase of fermentation (glycogen to pyruvate) the following enzymes are —SH enzymes: muscle phosphorylase, phosphoglucomutase, hexokinase, phosphoglyceraldehyde oxidase. In the second phase of fermentation (pyruvate to lactate or pyruvate to acetaldehyde + CO₂), the enzyme leading to alcohol fermentation, carboxylase, is an —SH enzyme, while the enzyme leading to lactate formation is not. In the oxidative phase of carbohydrate metabolism there is some evidence for considering hexose monophosphate oxidase an —SH enzyme, the evidence being inhibition by CuCl₂ and iodoacetate (reported by von Euler and Adler). Evidence has been presented here that pyruvate oxidation, pyruvate condensation, malate oxidation, and α -ketoglutarate oxidation require —SH enzymes. Succinate oxidation and yeast alcohol oxidation also require —SH enzymes. Among the phosphorylases which take part in the transphosphorylations necessary for the continuous fermentative and oxidative processes, the following are —SH enzymes: adenosinetriphosphatase (myosin) and myokinase. In short, so many steps in the the series of reactions occurring during carbohydrate metabolism require —SH enzymes, that it may be concluded that sulfhydryl enzymes occupy an important place in the breakdown and synthesis of glycogen.

SUMMARY

Reagents used for the detection of essential —SH groups in enzymes have been classified according to their affinity for these groups. Among the enzymes taking part in carbohydrate metabolism the following were inhibited by the —SH reagents: pyruvate oxidase, carboxylase, α -ketoglutarate oxidase, malate oxidase, adenosinetriphosphatase (myosin), and the enzymes for pyruvate condensation. Enzymes not inhibited by the —SH reagents were lactate oxidase, isocitrate oxidase, acid phosphatase, carbonic anhydrase, polyphenol oxidase, and catalase. Essential —SH groups were found only in the activating proteins (the proteins which combine with the substrate). Cytochrome oxidase and flavoproteins were not inhibited by —SH reagents.

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SULFHYDRYL ENZYMES IN FAT AND PROTEIN METABOLISM

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The large number of enzyme systems concerned with carbohydrate metabolism which require the presence of —SH groups in the molecule of the activating protein (dehydrogenase) made it imperative to determine whether the presence of these same groups was required in the case of enzymes concerned with the metabolism of fats and proteins. Very little is known regarding the necessity of —SH groups for the activity of fat oxidation enzymes. Green and coworkers (1) reported that β -hydroxybutyric dehydrogenase is inhibited by 0.03 M iodoacetate and arsenite, both —SH reagents of doubtful value. According to Yosii (2), an enzyme in adipose tissue responsible for the oxidation of higher fatty acids is inhibited by heavy metals (Ag, Cu, Hg), an indication that the enzyme might require —SH groups. The water-soluble enzyme extracted from liver by Konrad-Lang (3), which oxidizes stearate and palmitate, was not inhibited by iodoacetate. Much work has been done, however, on enzymes concerned with protein metabolism. Suffice it to recall that Perlzweig's discovery (4) of —SH groups in urease initiated the extensive experimental work on the detection of essential —SH groups in hydrolytic and proteolytic enzymes. Very little information is available on the necessity of —SH groups in other enzymes concerned with protein metabolism, the oxidation of amino acids, and transamination. Friedenwald and Herrmann (5) have demonstrated that amine oxidase is a sulfhydryl enzyme. We had reached the same conclusion in experiments performed previously to Friedenwald's report.

In this paper we present the results of a systematic investigation of the necessity of —SH groups for the activity of some enzymes concerned with the metabolism of fats and proteins. From these studies, and from those previously reported, it may be concluded that the presence of —SH groups in the protein component of a large number of enzymes is essential for activity.

The great lability of many of these —SH groups (as shown by the difficulty of preparing many of the —SH enzymes unless a reducing substance is present), the restoration of enzyme activity on addition of glutathione, and the almost universal presence of this tripeptide as an intracellular

constituent make plausible the suggestion that one of the functions of glutathione is that of maintaining the great number of the —SH enzymes at optimum activity. Glutathione would then be necessary for the metabolism of all foodstuffs (carbohydrates, fats, proteins), since many of the reactions of the metabolic cycle are catalyzed by —SH enzymes.

Sulfhydryl Enzymes in Fat Metabolism

There is, it seems, no field of enzymology more lacking in information than that of enzymes concerned with the metabolism of fats. Enzymes from liver or from bacteria oxidize saturated, high molecular weight fatty acids to the corresponding unsaturated fatty acids; some enzymes from plants and bacteria readily oxidize certain unsaturated fatty acids; and a number of bacteria readily oxidize saturated lower fatty acids. However, the components of the enzyme systems as well as the mechanisms of enzyme action are still unknown.

Stearate Oxidase—The enzyme for the oxidation of stearate was tested in the aqueous extract obtained according to the method of Konrad-Lang (3), and in suspensions of washed *Bacillus coli*.

The liver extract of Konrad-Lang produces activation of stearate in the presence of added adenosine triphosphate; a reversible dye must be used as the oxidizing agent. The time of methylene blue reduction was observed in Thunberg tubes kept in a vacuum at 38°. Complete reduction occurred in the control tubes in 11 minutes. In the presence of 1×10^{-4} M *p*-chloromercuribenzoate the activity of the enzyme was completely lost; with 1×10^{-3} M iodoacetate the reduction time was 14 minutes; with 1×10^{-3} M *p*-hydroxyphenylarsine oxide 75 per cent reduction occurred in 70 minutes. Since the dye reduction proceeds as a first order reaction, the degree of inhibition was calculated from the reduction time (Table I).

Washed suspensions of *Bacillus coli* oxidize stearate and oleate (6). The oxidation of stearate was completely inhibited by 5×10^{-5} M *p*-chloromercuribenzoate; 1×10^{-3} M *p*-carboxyphenylarsine oxide produced an inhibition of 77 per cent; 1×10^{-3} M iodoacetate inhibited by 47 per cent; and 2×10^{-3} M iodoacetamide gave an inhibition of 80 per cent. The oxidation of oleate was also inhibited by all these reagents to about the same extent as the inhibition of stearate; alloxan (1×10^{-2} M) inhibited by 33 per cent (Table II).

The oxidation of oleic acid by an enzyme extracted from peanuts was not affected by any of the —SH reagents tested.

Acetate Oxidation—The oxidation of acetic acid by yeast seems to start, according to Lynen (7), by a condensation of acetate with oxalacetate and formation of isocitric acid. The oxidation of isocitric acid would give α -ketoglutaric acid which in turn would be oxidized to succinic acid. Ace-

tate would thus enter the C₄-dicarboxylic acids series of catalysts. The oxidation of acetate by washed bakers' yeast was completely inhibited

TABLE I

Effect of —SH Reagents on Stearate Dehydrogenase from Rat Liver

Enzyme, 1 cc. of dialyzed rat liver extract + 1 cc. of 10^{-2} M sodium stearate + 0.25 cc. of phosphate buffer, 0.1 M, pH 7.4, containing 50 γ of adenosine triphosphate. Methylene blue, 0.15 cc. of 1:5000 solution.

Inhibitor	Concentration of inhibitor <i>M</i>	Reduction		Inhibition <i>per cent</i>
		<i>per cent</i>	<i>min.</i>	
Blank			∞	
None		100	11	
<i>p</i> -Chloromercuribenzoate	1×10^{-4}	100	∞	Complete
Iodoacetate	1×10^{-3}	100	14	11
<i>p</i> -Carboxyphenylarsine oxide	1×10^{-3}	75	70	78

TABLE II

Inhibition of Fatty Acid Oxidation by Bacillus coli communis

2.2 cc. of twice washed bacteria suspended in 0.04 M Ringer-phosphate solution, pH 7.4; substrate, 1 cc. of 1×10^{-2} M sodium salt; buffer, 0.05 M phosphate, pH 7.4, to 4 cc.; 38°; gas phase, air; duration of experiments, 40 minutes.

Inhibitor	Concentration of inhibitor <i>M</i>	Substrate	O ₂ consumption (blank subtracted)	Inhibition <i>per cent</i>
			<i>c.mm.</i>	
None		Stearate	46.8	
<i>p</i> -Carboxyphenylarsine oxide	1×10^{-3}	"	10.6	77.4
None		Oleate	57.4	
<i>p</i> -Carboxyphenylarsine oxide	1×10^{-3}	"	15.6	72.9
None		Stearate	55.0	
<i>p</i> -Chloromercuribenzoate	5×10^{-5}	"	0	100
None		Oleate	70.8	
<i>p</i> -Chloromercuribenzoate	5×10^{-5}	"	0	100
None		Stearate	59.5	
Iodoacetate	1×10^{-3}	"	31.4	47.3
Iodoacetamide	2×10^{-3}	"	11.8	80.2
None		Oleate	85.5	
Iodoacetate	1×10^{-3}	"	49.2	42.5
Iodoacetamide	2×10^{-3}	"	15.8	81.5
None		"	72.9	
Alloxan	1×10^{-2}	"	48.5	33.5

by *p*-chloromercuribenzoate (1.5×10^{-3} M) and by *p*-carboxyphenylarsine oxide (2×10^{-3} M). Since the oxidation of isocitric acid is not inhibited by these substances, it may be concluded that the condensation reaction

of acetate with oxalacetate, first phase of acetate oxidation, requires an —SH enzyme.

Corynebacterium creatinovorans strain NC, the soil bacteria discovered by Dubos and Miller (8), increases its ability to oxidize acetate when grown in a medium containing acetate as the sole source of carbon. The oxidation of acetate by these bacteria was completely inhibited by *p*-chloromercuribenzoate (5×10^{-4} M), 94 per cent by iodoacetamide (5×10^{-3} M), and 98 per cent by 3-amino-4-hydroxyphenyldichloroarsine hydrochloride

TABLE III

Effect of —SH Reagents on Acetate Oxidation by Bakers' Yeast, and by Corynebacterium creatinovorans

0.3 cc. of washed bakers' yeast (7.2 mg. dry weight) suspended in water; 1.0 cc. of 0.2 M PO_4 , pH 6.8; 0.3 cc. of 0.1 M sodium acetate; inhibitors and water to 3.0 cc.; 38°; gas phase, air; duration of experiments, 60 minutes. The —SH reagents were left in contact with the yeast for 10 minutes before the glutathione was added. Appropriate blanks were subtracted. The *Corynebacterium creatinovorans* was grown on acetate broth. 1 cc. of washed bacteria was used per vessel. Buffer, 0.02 M phosphate, pH 7.4; substrate, 0.01 M acetate; 38°; duration of experiments, 30 minutes.

Inhibitor and reactivator	Oxygen consumption		Inhibition
	Control	Inhibitor	
	c.mm.	c.mm.	per cent
1. Bakers' yeast			
<i>p</i> -Chloromercuribenzoate, 1.5×10^{-3} M	207	0	Complete
“ “ 1×10^{-4} M	203.8	73.4	64
<i>p</i> -Carboxyphenylarsine oxide, 2×10^{-3} M	207	5.9	97
“ “ 5×10^{-4} “	203.8	160.3	21
2. <i>Corynebacterium creatinovorans</i>			
<i>p</i> -Chloromercuribenzoate, 5×10^{-4} M	186.0	0	Complete
3-Amino-4-hydroxyphenyldichloroarsine hydrochloride, 2×10^{-4} M	195.4	4.4	98.5
Iodoacetamide, 5×10^{-3} M	186.0	11.8	93.7

(Table III). If oxidation of acetate by these bacteria follows the same path as in yeast, these experiments would be confirmatory of those with yeast.

β -Hydroxybutyrate Dehydrogenase—It was shown by Green that β -hydroxybutyrate dehydrogenase is inhibited by iodoacetate and arsenite, an indication that the enzyme requires —SH groups for activity. In agreement with this assumption we found a complete inhibition of this oxidation by *p*-chloromercuribenzoate (5×10^{-4} M) and *p*-carboxyphenylarsine oxide (5×10^{-4} M), and a large inhibition produced by iodoacetamide (72 per cent with 5×10^{-3} M iodoacetamide) (Table IV). In these

experiments the oxidation of β -hydroxybutyrate was measured by the O_2 uptake in the presence of diphosphopyridine nucleotide. The preparation contained the flavin dinucleotide and cytochrome system necessary for electron transfer to molecular oxygen.

Pancreatic Lipase—In an extensive study of various enzyme inhibitors on the hydrolysis of tripropionin by pancreatic lipase, Weinstein and Wynne (9) found that heavy metals (Cu^{++} , Fe^{+++} , Hg^{++}), and the acid halides, chloro-, bromo-, and iodoacetic acids, inhibited the enzyme; on the other hand KCN , $Na_2S_2O_4$, cysteine, and thioglycolic acid activated it. Since acid halides combine with both $-SH$ and $-NH_2$ groups at H^+ ion concentrations at which the experiments were conducted (10), these authors left unanswered the mechanism of inhibition. The experiments reported in

TABLE IV

Inhibition of β -Hydroxybutyrate Oxidase by $-SH$ Reagents

Enzyme, prepared according to Green (1), 3 cc.; substrate, 3.3×10^{-3} M sodium β -hydroxybutyrate; buffer, pyrophosphate, pH 7.4; 0.3 cc. of 0.5 per cent diphosphopyridine nucleotide; 38° ; duration of experiments, 25 minutes.

Inhibitor	Concentration of inhibitor	O_2 uptake	Inhibition
	<i>M</i>	<i>c.mm.</i>	<i>per cent</i>
None		76.3	
<i>p</i> -Chloromercuribenzoate	5×10^{-4}	0	Complete
<i>p</i> -Carboxyphenylarsine oxide	5×10^{-4}	0	"
None		66.0	
Iodoacetamide	5×10^{-2}	18.6	72

Table V were performed at pH 7.4. *p*-Chloromercuribenzoate (1×10^{-3} M) inhibited the enzyme by 38 per cent; *p*-aminophenyldichloroarsine hydrochloride (1×10^{-3} M) inhibited 62 per cent; and 3-amino-4-hydroxyphenyldichloroarsine hydrochloride (1×10^{-3} M) produced an inhibition of 52 per cent. Iodoacetamide (5×10^{-3} M) had no effect at all.

Sulphydryl Enzymes in Protein Metabolism

Hellerman (11) and Bersin (12) have dealt extensively with the $-SH$ enzymes of hydrolytic and proteolytic types belonging to this group. Almost no work has been done with the enzymes concerned with oxidation and transamination reactions.

d-Amino acid oxidase as prepared according to Krebs (13) oxidizes alanine quantitatively to pyruvic acid, because the H_2O_2 formed during the reaction is destroyed as soon as formed by the catalase present in the kidney extract. The enzyme purified by Warburg and Christian (14) contains no

TABLE V

Effect of —SH Reagents on Pancreatic Lipase

Experiment I, 2 cc. of enzyme containing 75 mg. of pancreatin; substrate, 4 cc. of cottonseed oil; buffer, 10 cc. of 0.5 M phosphate, pH 7.5; diluted with water to a final volume of 20 cc. After 3 hours shaking at room temperature, alcohol was added to stop the reaction. Titration with NaOH to phenolphthalein. Experiment II, 2 cc. of enzyme containing 100 mg. of pancreatin; substrate, 2 cc. of cottonseed oil; 0.003 M phosphate added to a final volume of 5 cc. Duration of experiment, 20 hours. Experiment III, 2 cc. of enzyme containing 100 mg. of pancreatin, in 0.005 M phosphate, pH 7.5; substrate, 2 cc. of corn oil; final volume, 5 cc.; duration of experiment, 6 hours.

Experiment No.	Inhibitor	Concentration of inhibitor	Fatty acid liberated	Inhibition
		<i>M</i>	<i>micromoles</i>	<i>per cent</i>
I	None		5220	
	<i>p</i> -Chloromercuribenzoate	1×10^{-3}	3250	38
	Iodoacetamide	5×10^{-3}	5160	0
II	None		2020	
	<i>p</i> -Aminophenyldichloroarsine hydrochloride	1×10^{-3}	760	62
III	None		702	
	3-Amino-4-hydroxyphenyldichloroarsine hydrochloride	1×10^{-3}	339	51.7

TABLE VI

Effect of —SH Reagents on d-Amino Acid Oxidase

Experiment I, 1 cc. of rat kidney extract in 2×10^{-2} M pyrophosphate, pH 8.3, + 0.05 M pyrophosphate to 3 cc.; alanine concentration, 1.7×10^{-1} M (blank values subtracted); time, 30 minutes. Experiment II, at the end of 30 minutes, 0.3 cc. of 3 M acetate buffer, pH 4.81, was added and pyruvate was determined with yeast carboxylase. Experiment III, 2 cc. of enzyme in 0.05 M pyrophosphate, pH 7.96; H₂O to 3 cc.; time, 30 minutes; in all experiments the alanine concentration was 1.7×10^{-1} M; 38°; gas phase, air.

Experiment No.	Inhibitor	Concentration	O ₂ uptake or pyruvate utilization	Inhibition
		<i>M</i>	<i>c.mm.</i>	<i>per cent</i>
I	None		138	
	<i>p</i> -Chloromercuribenzoate (O ₂ uptake measured)	1×10^{-4}	30.8	78
II	None		315.1	
	<i>p</i> -Aminophenyldichloroarsine hydrochloride (pyruvate utilization measured)	5×10^{-5}	34.3	90
III	None		127.6	
	Iodoacetamide (O ₂ uptake measured)	5×10^{-3}	124	None

catalase and thus the end-product of alanine oxidation is acetic acid, produced by oxidation of pyruvic acid by H_2O_2 . Krebs' preparation makes possible the determination of enzyme activity by two simple methods, O_2 consumption and pyruvate analysis. The oxidation of alanine by *d*-amino acid oxidase was largely inhibited by *p*-chloromercuribenzoate (78 per cent by 1×10^{-4} M *p*-chloromercuribenzoate) and by *p*-aminophenyldichloroarsine hydrochloride (90 per cent by the 5×10^{-5} M arsenical). Iodoacetamide, however, had no effect at a concentration of 5×10^{-3} M (Table VI). The O_2 consumption was measured in experiments with the first and third inhibitor; in those with the arsenical, the activity was measured by determining pyruvate formation. This inhibition is due exclusively to mercaptide formation with the $-\text{SH}$ groups of the protein moiety. When the enzyme was purified and the flavin dinucleotide was split from the protein according to Negelein and Brömel (15), addition of a large excess of flavin dinucleotide to the protein did not reverse the inhibition of the enzyme. The inhibition disappeared on addition of further amounts of the protein from which the prosthetic group, flavin dinucleotide, had been separated (Fig. 1).

Transaminase—The essential $-\text{SH}$ groups in this enzyme were detected with a heart extract prepared according to Cohen (16). Two reactions were studied in these experiments: (1) pyruvate + *l*-glutamate $\rightleftharpoons$ α -ketoglutarate + *l*-alanine; (2) oxalacetate + *l*-glutamate $\rightleftharpoons$ α -ketoglutarate + *l*-aspartate. In the first reaction, transamination was measured by the determination of pyruvate disappearance (yeast carboxylase); in the second reaction, by determination of the aspartate formation (Cohen's method (17)). The formation of alanine by transamination of pyruvate from *l*-glutamate was inhibited 81 per cent by *p*-carboxyphenylarsine oxide (1×10^{-3} M), and the formation of *l*-aspartate by transamination of oxalacetate from *l*-glutamate was inhibited 49 per cent by *p*-chloromercuribenzoate (1×10^{-3} M). As in the case of *d*-amino acid oxidase, iodoacetamide had no effect on transaminase (Table VII).

Amine Oxidases—Friedenwald and Herrmann (5) reported that tyramine oxidase was inhibited by *p*-chloromercuribenzoate. The experiments reported in Table VIII are in agreement with the conclusions of Friedenwald and Herrmann that amine oxidase is an $-\text{SH}$ enzyme. The enzyme used in these experiments was prepared according to Blaschko *et al.* (18). The oxidation of tyramine was inhibited 82 per cent by *p*-chloromercuribenzoate (1×10^{-3} M), 76 per cent by *p*-carboxyphenylarsine oxide, 77 per cent by *p*-aminophenyldichloroarsine hydrochloride (1×10^{-3} M), and 33 per cent by iodoacetamide (5×10^{-3} M). The oxidation of adrenalin was inhibited 44 per cent by 3-amino-4-hydroxyphenyldichloroarsine hydrochloride (5×10^{-4} M).

Diamine oxidase prepared from kidney according to Laskowski (19) was unaffected by *p*-chloromercuribenzoate and by arsenicals (Table VIII).

l-Glutamic Dehydrogenase—The activating protein of *l*-glutamate oxidase is contained in water extracts of pig liver acetone powder prepared according to Dewan (20). When the diphosphopyridine nucleotide is split off, the activity of the enzyme can be measured by following spectrophotometrically

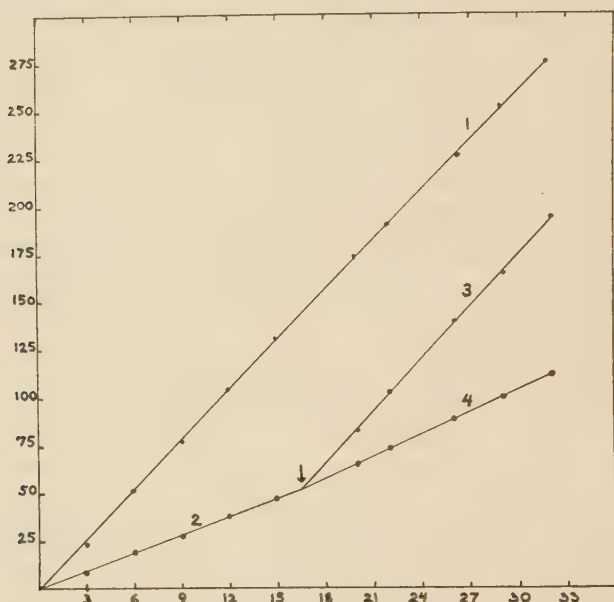


FIG. 1. Combination of the arsenoxide with the protein moiety of *d*-amino acid oxidase. Abscissa, time in minutes; ordinate, c.mm. of O₂ uptake. Curve 1, control; Curve 2, 1×10^{-4} M *p*-aminophenyldichloroarsine hydrochloride; Curve 3, same plus 1 cc. of activating protein; Curve 4, 1×10^{-4} M arsenical plus 500 γ of flavin dinucleotide. In the main vessel, 1 cc. of activating protein solution; 500 γ of crude flavin adenine dinucleotide in 0.2 cc.; 0.3 cc. of 0.55 M *dl*-alanine; 1 cc. of 0.05 M pyrophosphate buffer, pH 8.3; 0.5 cc. of arsenical or water. In the side arm, 1 cc. of activating protein or 500 γ of flavin dinucleotide in 1 cc. The contents of the side arm were tipped in at the end of 16.5 minutes. Temperature 38°; equilibration time, 10 minutes.

the reduction of added diphosphopyridine nucleotide, or by measuring the O₂ consumption with pyocyanine as the oxidizing catalyst. *p*-Chloromercuribenzoate (5×10^{-4} M) and 3-amino-4-hydroxyphenyldichloroarsine hydrochloride (1×10^{-3} M) inhibited completely the activity of the enzyme. Again, iodoacetamide (5×10^{-3} M) had no effect at all (Table IX).

Pepsin and *trypsin* were not inhibited by *p*-chloromercuribenzoate (1×10^{-3} M) or by the arsenicals.

Reactivation of —SH Enzymes with Glutathione—Conclusive evidence of the necessity of —SH groups for enzyme activity is obtained when the

TABLE VII

Effect of —SH Reagents on Transaminase

Experiment I, enzyme in 1 per cent KHCO_3 , 2 cc.; substrates, 0.5 cc. of 0.12 M pyruvate and 0.2 cc. of 0.3 M *l*-glutamate; time, 90 minutes; 38°. Pyruvate disappearance was determined with yeast carboxylase. Experiments II and III, enzyme, 3 cc.; substrates, 1 cc. of 0.06 M glutamate and 0.3 cc. of 0.2 M oxalacetate; time, 90 minutes; 38°. Aspartate formation was determined with chloramine-T.

Experiment No.	Inhibitor	Concentration	Pyruvate disappearance or aspartate formation	Inhibition
		M	c.mm.	per cent
I	None		514	
	<i>p</i> -Carboxyphenylarsine oxide	1×10^{-3}	95	81.5
	“ “	1×10^{-4}	292	43.2
II	None		1230	
	<i>p</i> -Chloromercuribenzoate	1×10^{-3}	632	48.6
III	None		748	
	Iodoacetamide	5×10^{-3}	747	None

TABLE VIII

Effect of —SH Reagents on Amine Oxidases

Experiment I, enzyme, from guinea pig liver, 2 cc.; buffer, 0.05 M phosphate, pH 7.3; tyramine concentration, 0.036 M; duration of experiments, 100 minutes. Experiment II, enzyme, 3 cc.; buffer, 0.06 M phosphate, pH 7.3; adrenalin concentration, 0.0125 M; duration of experiments, 40 minutes. Experiment III, enzyme, from pig kidneys, in 0.2 M PO_4 , pH 7.2; substrate, 0.0066 M histamine; duration of experiments, 270 minutes.

Experiment No.	Enzyme-substrate	Inhibitor	Concentration	O ₂ uptake	Inhibition
			M	c.mm.	per cent
I	Monoamine oxidase-tyramine	None		188.2	
		<i>p</i> -Chloromercuribenzoate	1×10^{-3}	33.4	82.3
		<i>p</i> -Carboxyphenylarsine oxide	1×10^{-3}	45.2	76.0
		<i>p</i> -Aminophenyldichloroarsine hydrochloride	1×10^{-3}	43.2	77.0
		Iodoacetamide	5×10^{-3}	125.2	33.3
		None		119.1	
II	Adrenalin	3-Amino-4-hydroxyphenyldichloroarsine hydrochloride	5×10^{-4}	66.7	44
		None			
III	Diamine oxidase-histidine	None		220	
		<i>p</i> -Carbamylphenylarsine oxide	1×10^{-3}	210	None

enzyme, inhibited by a mercaptide-forming compound, is brought back to full activity on addition of thiols. Reactivation experiments performed with the —SH enzymes discussed in this paper are reported in Table X.

Acetate oxidation by yeast, completely inhibited by *p*-chloromercuribenzoate, was reactivated 75 per cent on addition of glutathione. Acetate oxidation by *Corynebacterium creatinovorans*, inhibited by 3-amino-4-hydroxyphenyldichloroarsine hydrochloride, was reactivated 91 per cent on addition of glutathione (ratio of glutathione to arsenical, 20:1). The activity of β -hydroxybutyrate dehydrogenase, inhibited completely by 5×10^{-4} M *p*-chloromercuribenzoate, was completely restored on addition of 5×10^{-3} M glutathione. In fact, the —SH groups of this enzyme are so labile that in order to obtain preparations of optimal activity it would be necessary to perform the extraction in the presence of glutathione or cysteine. The activity of lipase (commercial pancreatin), inhibited by an organic arsenical, was partially restored (45 per cent reactivation) on addi-

TABLE IX

Effect of —SH Reagents on l-Glutamate Oxidase (Activating Protein or Dehydrogenase)

Enzyme (water extract of pig liver acetone powder; diphosphopyridine nucleotide (DPN) split off by acidification at pH 4.6; resuspended in 0.1 M phosphate buffer, pH 7.3) 1 cc.; DPN, 2 γ of 40 per cent purity in 0.3 cc.; *l*-glutamate, 0.25 M, 0.5 cc.; inhibitor or H₂O, 0.4 cc.; 0.1 M phosphate, pH 7.4, 1.8 cc.; 24.5°; rate of reduction measured at 3450 A, the substrate being added at 0 time.

Inhibitor	Concentration	Half reduction of DPN	Inhibition
	M	min.	per cent
None		1.88	
<i>p</i> -Chloromercuribenzoate	5×10^{-4}	∞	Complete
Iodoacetamide	5×10^{-3}	1.95	None
3-Amino-4-hydroxyphenyldichloroarsine hydrochloride	1×10^{-3}	∞	Complete

tion of glutathione. The activity of *d*-amino acid oxidase, completely inhibited by 3-amino-4-hydroxyphenyldichloroarsine hydrochloride, was completely restored on addition of glutathione (10 times the concentration of the inhibitor). It has been reported by Friedenwald and Herrmann (5) that tyramine oxidase inhibited by *p*-chloromercuribenzoate is only partially restored on addition of glutathione and that complete reactivation is reached in the presence of cyanide. Addition of cyanide diminishes the amount of *p*-chloromercuribenzoate present in the preparation owing to the formation of a complex compound between cyanide and *p*-chloromercuribenzoate; diminished inhibition will be the result of cyanide addition. In fact, Friedenwald and Herrmann report 85 per cent inhibition with 0.01 M *p*-chloromercuribenzoate (30 micromoles presumably in 3 cc. of fluid); in the presence of 0.005 M cyanide (1 mg. of KCN presumably in

TABLE X
Reactivation of *Sulphydryl Enzymes*

The conditions of the experiments were similar to those described in the preceding tables.

Enzyme	Inhibitor	Inhibition <i>per cent</i>	Thiol	Reactivation <i>per cent</i>
Acetate oxidation, bakers' yeast	<i>p</i> -Chloromercuribenzoate, 1.5×10^{-3} M	Complete	Glutathione, 1×10^{-2} M	75
“ <i>Corynebacterium creatinovorans</i>	3-Amino-4-hydroxyphenyldichloroarsine hydrochloride, 2×10^{-4} M	98.5	“ 4×10^{-3} “	91
β -Hydroxybutyrate oxidase	<i>p</i> -Chloromercuribenzoate, 5×10^{-4} M	Complete	“ 5×10^{-3} “	Complete
Lipase (pancreatic)	3-Amino-4-hydroxyphenyldichloroarsine hydrochloride, 1×10^{-3} M	52	“ 1×10^{-3} “	44.5
<i>d</i> -Amino acid oxidase	3-Amino-4-hydroxyphenyldichloroarsine hydrochloride, 1×10^{-3} M	91	“ 1×10^{-3} “	Complete
Monoamine oxidase	<i>p</i> -Chloromercuribenzoate, 1×10^{-3} M	83	“ 1×10^{-2} “	30
	<i>p</i> -Carbamylphenylarsine oxide, 1×10^{-3} M	76	“ 1×10^{-2} “	Complete
	<i>p</i> -Aminophenyldichloroarsine hydrochloride, 1×10^{-3} M	77	“ 1×10^{-2} “	“
Transaminase	<i>p</i> -Chloromercuribenzoate, 1×10^{-3} M	49	H ₂ S, 1×10^{-2} M	“
	“ 1×10^{-3} “	49	Cysteine, 5×10^{-3} M	97
<i>l</i> -Glutamate oxidase	“ 5×10^{-4} “	Complete	Glutathione, 5×10^{-3} M	69
	3-Amino-4-hydroxyphenyldichloroarsine hydrochloride, 1×10^{-3} M	“	“ 1×10^{-2} “	Complete

3 cc. of fluid) the inhibitions dropped to 35 per cent. The "complete reactivation" on addition of glutathione is thus due to diminished inhibition. The tyramine oxidase preparation used by us, when inhibited by *p*-chloromercuribenzoate (83 per cent inhibition), was reactivated 30 per cent on addition of glutathione (ratio of glutathione to inhibitor, 10:1); on the other hand, the inhibition produced by 1×10^{-3} M *p*-carbamylphenylarsine oxide and 1×10^{-3} M *p*-aminophenyldichloroarsine hydrochloride was completely abolished on addition of glutathione at the usual ratio of 10:1. Transaminase, inhibited by *p*-chloromercuribenzoate, was completely reactivated on addition of H₂S or cysteine. The activity of *l*-glutamate dehydrogenase, inhibited by *p*-chloromercuribenzoate and 3-amino-4-hydroxyphenyldichloroarsine hydrochloride, was completely restored on addition of glutathione (Table X).

Rôle of Glutathione—Since the discovery of glutathione in 1921 by Hopkins and the establishment of its almost universal distribution in the intracellular fluid of animal tissues, many hypotheses have been formulated regarding the biological rôle of this tripeptide. It has been shown by other investigators, as well as in the experiments reported here and in the preceding paper (21), that glutathione produces reactivation of —SH enzymes when the —SH groups have been rendered ineffective by oxidation or by mercaptide formation. Thus, in the preceding paper (21), it was shown that sulfhydryl groups are essential for the activity of a large number of enzymes concerned with the metabolism of carbohydrate. Phosphorylases, oxidases, carboxylases, and decarboxylases required —SH groups for enzyme activity. We have presented in this paper observations on another large group of enzymes; namely, those concerned with the metabolism of fats and proteins, for the activity of which the presence of —SH groups in the enzyme protein molecule is required. If to these enzymes we add those found previously, the result is an indeed impressive array of sulfhydryl enzymes. In some cases (urease) the essential —SH groups (sluggish —SH groups) are more or less protected from the action of oxidizing agents; in others (muscle phosphorylase, pyruvate oxidase) the essential —SH groups are extremely labile, being oxidized by such sluggish oxidizing agents as molecular oxygen (freely reacting —SH groups). The continuous production of oxidizing agents in the cell will tend to inhibit the activity of the —SH enzymes. It is therefore reasonable to assume that the main rôle of glutathione in cellular systems is that of continuous reactivation of the —SH enzymes. Of course, the degree of activity of these enzymes is determined, among other factors, by the oxidation-reduction potential of the cell; *i.e.*, by the concentration of glutathione, of ascorbic acid, of oxidants, and of hydrogen ions, etc.

SUMMARY

Among the enzymes concerned with the metabolism of fats, the following have been found to require the presence of —SH groups in the activating protein: liver stearate oxidase, *Bacillus coli* stearate and oleate oxidase, β -hydroxybutyric dehydrogenase, acetate oxidase, and pancreatic lipase. The enzyme that oxidizes oleic acid in peanuts contained no essential —SH groups. Among the enzymes for protein metabolism, the following were found to be —SH enzymes: *d*-amino acid oxidase, transaminase, *l*-glutamic dehydrogenase, and monoamine oxidase. Diamine oxidase, pepsin, and trypsin contained no essential —SH groups. The enzymes were inhibited by alkylating agents (iodoacetamide) and by mercaptide-forming agents (*p*-chloromercuribenzoic acid and organic arsenicals). Addition of glutathione at a ratio of 10:1 of inhibitor restored the activity of these enzymes. The rôle of glutathione in cellular activities is discussed on the basis of these experiments.

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